**Probe Report**

**Title:** A small molecule activator of KCNQ2 and KCNQ4 channels
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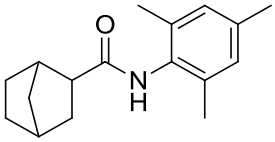
**Assigned Assay Grant #:** 1 R03 DA027716-01
**Screening Center Name & PI:** Johns Hopkins Ion Channel Center, Min Li
**Chemistry Center Name & PI:** Vanderbilt Specialized Chemistry Center, Craig Lindsley
**Assay Submitter & Institution:** Min Li, Johns Hopkins University
**PubChem Summary Bioassay Identifier (AID):** 2258

**Abstract:**
ML213 was identified following a high throughput fluorescent screen of the Molecular Libraries Small Molecule Repository (MLSMR) library and structure activity relationship (SAR) studies using fluorescent and electrophysiological assays to determine potency and selectivity of test compounds. ML213 is a potent activator of potassium voltage-gated channel, KQT-like subfamily, member 2 (KCNQ2) ($K_v$7.2, $EC_{50}$ = 230 nM) and KCNQ4 ($K_v$7.4, $EC_{50}$ = 510 nM) and selective against the other members of the KCNQ family of ion channels (KCNQ1, KCNQ3 and KCNQ5). ML213 shifts the voltage-dependence of KCNQ2 opening in a concentration dependent manner with a comparable half maximal effective concentration ($EC_{50}$) value and produces a maximal hyperpolarizing shift of 37 mV. In addition, ML213 has been tested in 247 assays performed within the MLPCN network and was active in only one assay that was not dependent on KCNQ channels. ML213 was also tested at Ricerca's Lead Profiling Screen (binding assay panel of 68 G protein coupled receptors [GPCRs], ion channels and transporters screened at 10 μM), and was found to not significantly bind with any of the 68 assays conducted. This is consistent with its high selectivity. ML213 was stable in aqueous media (>24 hour half life). However, it exhibited poor metabolic stability when incubated with rat or human liver microsomes, predicting rapid clearance after systemic exposure. Although there are several reported KCNQ2/3 activators in the primary literature, ML213 (CID 3111211) has several unique features, especially being the first KCNQ2 and KCNQ4 selective probe compound and, as such, ML213 will be an important tool for understanding the roles of these channels in regulating neuronal excitability. The probe may be used for investigations of the roles of KCNQ2 and KCNQ4 channels in epileptogenesis in mechanistic cellular studies and tissue slice experiments. Its metabolic properties may be improved with a more extensive and systemic SAR campaign.

## Probe Structure & Characteristics:



N-mesitylbicyclo[2.2.1]heptane-2-carboxamide, **ML213**

| CID/ML# | Target Name | $EC_{50}$ (nM) [SID, AID] | Anti-target Name(s) | $IC_{50}/EC_{50}$ (μM) [SID, AID] | Fold Selective | Secondary Assay(s) Name: $IC_{50}/EC_{50}$ (nM) [SID, AID] |
|---|---|---|---|---|---|---|
| CID 3111211/ ML213 | KCNQ2_Act _Tl | 359 [SID 103073346, AID 493037] | KCNQ1_Act_Tl | >30 [SID 103073346, AID 504417] | >80 | KCNQ2_Act_IWS: 368 [SID 103073346, AID 493038] |
| | | | KCNQ1/KCNE1-Act_Tl | >30 [SID 103073346, AID 504418] | >80 | |
| | | | KCNQ3_Tl | >30 [SID 103073346, AID 493047] | >80 | |
| | | | KCNQ4_Act_Tl | 2.4 [SID 103073346, AID 504416] | 6.7 | |
| | | | KCNQ5_Act_Tl | >30 [SID 103073346, AID 493046] | >80 | |
| | | | Kir2.1 counterscreen_Tl | >10 [SID 47200766, AID 2345] | >27 | |
| | KCNQ2_Act _IWS | 230 [SID 104223736, AID 493038] | KCNQ1_Act_IWS | >30 [SID 103073346, AID 493044] | >80 | KCNQ2_act_IW_W236 L: >25,000 [SID 47200766, AID 2558] |
| | | | KCNQ1/KCNE1_ Act_IWS | >30 [SID 103073346, AID 493042] | >80 | |
| | | | KCNQ4_Act_IWS | 0.51 [SID 103073346, AID 493043] | 2.2 | |
| | KCNQ2/KC NQ3_Act_I WS | 366 [SID 103073346, AID 493039] | | | | |

## Recommendations for scientific use of the probe:

This probe (ML213, CID 3111211) can be used to investigate the role of selective KCNQ2 and KCNQ4 activation *in vitro* and, potentially, *in vivo* studies. ML213 is a potent activator of KCNQ2 ($K_v$7.2, $EC_{50}$ = 230 nM) and KCNQ4 ($K_v$7.4, $EC_{50}$ = 510 nM) and selective against the

other members of the $K_v7$ family of ion channels ($K_v7.1$, KCNQ1; $K_v7.3$, KCNQ3; $K_v7.5$, KCNQ5). In addition, ML213 has been tested in 247 assays performed within the MLPCN network and was active in only one assay (Cycloheximide Counterscreen for Small Molecule Inhibitors of Shiga Toxin [Primary Screening; AID 2314]) that was not dependent on KCNQ2. And lastly, ML213 was tested at Ricerca's (formerly MDS Pharma's) Lead Profiling Screen (binding assay panel of 68 GPCRs, ion channels and transporters screened at 10 µM), and was found to not significantly bind with any of the 68 assay targets conducted (no inhibition of radio ligand binding > 50% at 10 µM). Although there are several reported KCNQ2/3 activators in the primary literature, ML213 (CID 3111211) represents the first KCNQ2 and KCNQ4 selective probe compound and as such, ML213 will be an important tool for understanding the roles of these channels in regulating neuronal excitability. The probe may be used for investigations of the roles of KCNQ2 and KCNQ4 channels in epileptogenesis in mechanistic cellular studies and tissue slice experiments.

## 1 Introduction

The KCNQ ($K_v7$) family of channels are six transmembrane domain voltage gated $K^+$ channels consisting of five members ($K_v7.1$ – $K_v7.5$) (*1*). In order to form functional channels, four $K_v7$ subunits are required, leading to increased complexity in composition and function of this family of channels (*2*). It is known that all five channel subunits can form homomeric channels *in vitro*, however, there are limitations on the subunit combinations that form heteromeric channels. In contrast with other $K_v$ channel families, KCNQ (or $K_v7$) channels commonly display activation at voltages close to neuronal resting membrane potentials and are regulated by GPCR signaling, notably by muscarinic receptors (*3*). Thus, KCNQ channels are critical for setting up the excitation threshold of action potentials. Among them, $K_v7.2$ – $K_v7.5$ are predominantly expressed (in rodents) in the peripheral and central nervous system, including hippocampal cells, cortical cells, and dorsal root ganglion. The $K_v7.1$ (or KCNQ1) is expressed in cardiac tissue and peripheral epithelial and smooth muscle cells (Table 1) (*2*).

**Table 1.** Expression of $K_v7$ channels in various tissues.

| $K_v7.x$ | KCNQ | Primary Distribution |
|---|---|---|
| $K_v7.1$ | KCNQ1 | Cardiac Tissue, Peripheral Epithelial, Smooth Muscle Cells |
| $K_v7.2$ | KCNQ2 | Peripheral and central nervous system |
| $K_v7.3$ | KCNQ3 | Peripheral and central nervous system |
| $K_v7.4$ | KCNQ4 | Auditory and vestibular system |
| $K_v7.5$ | KCNQ5 | Peripheral and central nervous system |

Voltage-gated potassium ($K^+$) channels are critical for neuronal function in excitable tissues such as brain and heart. They are also found in many non-excitable tissues and are important for functions in these tissues including hormone secretion, oxygen-sensing and immune responses. There are more than 100 genes in the human genome encoding different but homologous potassium channels. Voltage-gated $K^+$ channels, as exemplified by the Shaker $K^+$

channel, share considerable sequence similarity. The presumed structural similarity is supported by several available atomic structures of bacterial and mammalian $K^+$ channels, KcsA and $K_v1.2$ (*4, 5*). Isolation and characterization of bioactive chemical probes could form an important pharmacological foundation, providing insights into the function and role of structural components and also of the roles of specific channels in cell, tissue and organismal function. Because of the considerable sequence homology, channel blockers (or activators) specific for certain isoforms or oligomers are valuable reagents both for investigating channel function and for developing therapeutics.

A growing body of evidence now exists supporting the premise that neuronal KCNQ channels represent interesting targets for the treatment of diseases involving altered neuronal excitability, such as epilepsy and chronic pain. This evidence includes genetic studies (mutations in KCNQ2 and KCNQ3 cause benign familial neonatal convulsions (BFNC), a rare form of epilepsy (*6-8*)), the distribution of these channels in the CNS, and pharmacological experiments (*2, 9*). Supported by this data, a number of groups have initiated efforts to develop novel KCNQ activators (*2, 9-11*). The preceding references describe background studies and prior art in much detail; in this manuscript we will present an overview of the compounds best described in the available literature.

Retigabine (D-23129, **1**) (**Figure 1**) is first compound identified as a KCNQ activator (*12, 13*). Retigabine was originally developed as a modulator of GABA-ergic signaling; however, it was found to have little effect on the GABA pathway. Despite its poor effects on the GABA pathway, retigabine was found to have potent anti-convulsant activity (*12-14*). Following these initial studies, it was determined that retigabine was an activator of KCNQ2/3 potassium channels (*13, 15*), which has been followed up with extensive studies determining the $EC_{50}$ (1.6 μM) for activation of these channels (*16*). Retigabine potently enhances KCNQ2/3 currents by inducing a leftward shift in the voltage-dependence of channel activation. Retigabine's robust anti-convulsant activity in a number of seizure models may be due to the widespread distribution of KCNQ2/3 in the CNS and the molecule's robust activation of KCNQ2/3 channels. Retigabine has progressed into clinical development for the treatment of partial-onset epilepsies (*17*) and is currently in Phase III clinical trials and represents a novel mechanism of action as an anti-convulsant (*18*). Due to these clinical results and the pharmacological activity of retigabine, several structurally related compounds have been synthesized based on the retigabine scaffold. However, retigabine's application may be limited by modest potency and several other potentially undesirable characteristics including its broad action on KCNQ2, KCNQ3, KCNQ4, and KCNQ5 channels. Determination of the therapeutic utility of KCNQ activators as anticonvulsants must await additional clinical data and, possibly, more selective and potent compounds.

In 2001, Bristol-Meyers Squibb disclosed BMS-204352, **2**, a fluorinated oxindole as an agonist of calcium-activated potassium channels for treatment of stroke (Figure 1) (*19*). Subsequent studies revealed that BMS-204352 was an activator of KNCQ4 and KCNQ5 with equal potency ($EC_{50}$ = 2.4 μM) (*20-22*). In a more recent publication, it has been shown that BMS-204352 is a

non-selective $K_v7.x$ (KCNQx) activator with similar potencies against KCNQ2, KCNQ2/3, KCNQ4, KCNQ3/4, and KCNQ5 relative to retigabine (*23, 24*). BMS-204352 progressed into clinical trials for patients with acute stroke; however, it was reported that BMS-204352 failed to show superior efficacy compared to placebo (*23*).

More recently, Bristol-Myers Squibb has disclosed a series of acrylamides, **(*S*)-3** and **(*S*)-4**, as KCNQ2 potassium channel openers (*25-28*). SAR studies revealed that the (*S*)-configuration of the (1-phenyl)ethyl moiety, the acrylamide, and the free N-H are vital to the activity (**Figure 1**). Compound **3** was shown to have an $EC_{50}$ of 3.2 μM against KCNQ2 with a maximal % control of 163% (*25*). In addition, **(*S*)-3**, was shown acceptable *in vivo* PK properties and to be efficacious in a model of cortical spreading depression (CSD), suggesting KCNQ2 activators have the potential for treatment of CNS disorders characterized by hyperexcitability (*27*). An improved compound, **(*S*)-4**, was shown to be one of the first compounds to display sub-micromolar activity against KCNQ2 ($EC_{50}$ = 0.063 μM) (*27*). Compound **(*S*)-4** is very similar to **(*S*)-3** in that it also contains the (*S*)-configuration of the (1-phen)ethyl moiety, the acrylamide and the free NH; however, the right hand portion was cyclized to include a bicycle with a 2*H*-benzo[1,4]oxazine moiety. This compound was shown *in vitro* to reduce spontaneous neuronal discharges in rat hippocampal slices (*28*), again lending support for KCNQ2 activators as potential treatments for disorders characterized by neuronal hyperexcitability. Unfortunately, no further details are currently available concerning the selectivity of these compounds, other than that these compounds activate other members of the KCNQ family. Thus, the search for more selective KCNQ activators has still not been realized.

Lastly, Icagen, Inc. has published extensively on two novel scaffolds displaying KCNQ2/3-selective activation (**Figure 1**) (*29-31*). The first scaffold ICA-27243 ,**5**, was shown to be a sub-micromolar activator of KCNQ2/3 ($EC_{50}$ = 0.4 μM), with selectivity over KCNQ4 ($EC_{50}$ = 9.7 μM) and KCNQ3/5 (>30 μM). ICA-27243 is a heterobiaryl amide which represents a novel structural class of KCNQ activators. Furthermore, ICA-27243 has been shown to be orally bioavailable and active in multiple seizure models with efficacy observed as low as 1 mg/kg PO (*30*). In addition, ICA-27243 did not show any evidence for tolerance, dependence, or cognition impairment further validating KCNQ2/3 activators as attractive anti-epileptic drugs. The most recent disclosure by Icagen, Inc. details the discovery and SAR development of another novel structural class of selective KCNQ2/3 activators (**6**, Figure 1) (*31*). The researchers, starting with a non-selective HTS hit, were able to discover compound **6**, a benzothiazole hydrazine carboxylate, with nanomolar activity against KCNQ2/3 ($EC_{50}$ = 0.095 μM). Although very little selectivity data was presented, the researchers state that **6** is selective against a Cerep receptor binding panel and against KCNQ1/ KCNE1. No data has been presented against other subtypes. Hence, the overall selectivity profile remains largely unknown (or undisclosed). Compound **6** was also shown to be active in two *in vivo* anti-convulsant models ($ED_{50}$ < 10 mg/kg).

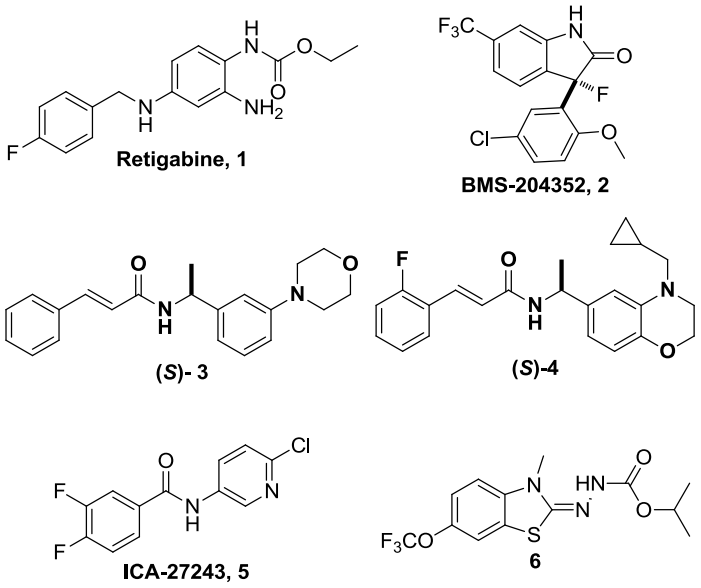
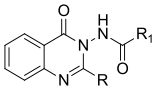
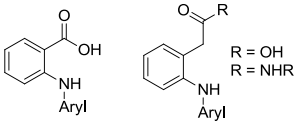



**Figure 1.** Recently reported KCNQ activators.

Twelve US patents issued prior to 2011 describe KCNQ channel activators (**Table 2**). Biological data on the effects of these compounds on different KCNQ channels are not fully available in these patents; however, most of the patents cover those compounds that have been described in the primary literature (vide supra). An additional class of KCNQ2 activators is described in US Patents 7,741,332 and 7,223,768 that were not described in the scientific papers, but no detailed descriptions of the effects of these compounds on specific KCNQ channels are presented.

**Table 2.** List of US patents issued prior to 2011 that claim compounds activating KCNQ channels.

| Patent number | Company | Target channel | Chemical class | Representative compound |
|---|---|---|---|---|
| 6,326,385 | Icagen | KCNQ2/Q3 | N-aryl benzamide | |
| 6,372,767 | Icagen | KCNQ2 | Benzanilides and 2-substituted-5-aminopyridines | |
| 6,495,550 | Icagen | KCNQ2 | Pyridine-substituted benzanilides | ICA-27243 analogs |
| 6,989,398 | Icagen | KCNQ2 | Benzanilides | |
| 6,605,725 | Icagen | KCNQ2 | Benzanilides | |
| 6,737,422 | Icagen | KCNQ2 | Benzanilides | |
| 6,593,349 | Icagen | KCNQ2 | Bisarylamines | |
| 7,741,332 | Icagen | KCNQ2 | Fused ring heterocycles |  |
| 7,223,768 | Icagen | KCNQ2 | Fused ring heterocycles | |
| 6,469,042 | BMS | KCNQ2, KCNQ2/Q3 | Fluoro oxindole derivatives | |
| 6,855,829 | BMS | KCNQ2, KCNQ5 | 3-fluoro-2-oxindole and 2,4-disubstituted pyrimidine-5-carboxamide | BMS-204352 analogs |
| 7,632,866 | Tel Aviv U. | KCNQ2/3, Q1, Q1/E1 | Derivatives of N-phenylanthranilic acid and 2-benzimidazolone as potassium channel and/or neuron activity modulators |  |

ML213 exhibits sub-micromolar activation of KCNQ2 and KCNQ4 channels, but in contrast to other described KCNQ activators, ML213 exhibits superior selectivity for activating other KCNQ channels (>80-fold selective).

## 2 Materials and Methods

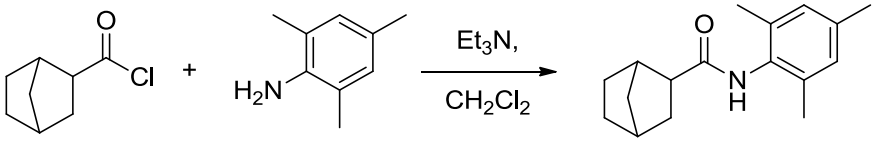
- CHO-KCNQ2 cell line
- Parental CHO cell line
- CHO-KCNQ1 cell line
- CHO-KCNQ1/KCNE1 cell line
- HEK-Kir2.1 cell line
- CHO-KCNQ2-W236L cell line
- CHO-K1, KCNQ3 expression vector
- CHO-K1, KCNQ4 cell line
- CHO-K1, KCNQ5 expression vector
- FluxOR dye kit (Invitrogen, F10017)
- BD Biocoat, Poly-D-Lysine coated, black/clear bottom 384-well assay plates (Fisher, 356936)
- Hamamatsu FDSS 6000 fluorescent plate reader
- Population Patch Clamp patch plates from Molecular Devices

### 2.1 Assays

- KCNQ2 potassium channel cell-based FDSS $Tl^+$ flux assay (see methods in 6.1 Appendix 1). The $Tl^+$ flux assay has been used for the following AIDs.
- HTS for KCNQ2 activators: AID 2239
- Confirmatory screen for KCNQ2 activators: AID 2287
- Counter screen for KCNQ2 activators: AID 2282
- Specificity screen against KCNQ1 for KCNQ2 activators: AID 2283
- Specificity screen against Kir2.1 for KCNQ2 activators: AID 2345
- Confirmatory dose response assay for KCNQ2 activators: AID 493037
- Specificity dose response assay against KCNQ1 channel: AID 504417
- Specificity dose response assay against KCNQ3 channel: AID 493047
- Specificity dose response assay against KCNQ4 channel: AID 504416
- Specificity dose response assay against KCNQ5 channel: AID 493046
- Specificity dose response assay against KCNQ1/KCNE1 channel: AID 504418
- KCNQ2 potassium channel IonWorks electrophysiology assay (see methods in 6.2 Appendix 2). The IonWorks electrophysiology assay has been used for the following AIDs.
- KCNQ2-W236L mutant test for KCNQ2 activators: AID 2558

- Dose response assay for KCNQ2-W236L active compounds on CHO-KCNQ2 cell line: AID 2603
- Dose response assay for KCNQ2 activators: AID 2548
- Dose response assay for SAR compounds (Scaffold-1): AID 2654
- Dose response assay for SAR compounds(Scaffold-2): AID 493113
- Dose response assay for KCNQ2 activators: AID 493038
- Dose response assay for KCNQ2/KCNQ3 activators: AID 493039
- Specificity dose response assay against KCNQ1 channel: AID 493044
- Specificity dose response assay against KCNQ4 channel: AID 493043
- Specificity dose response assay against KCNQ1/KCNE1 channel: AID 493042

### 2.2 Probe Chemical Characterization




Probe compound **ML213** (CID: 3111211, SID: 104223736) was prepared according to the above scheme and provided the following characterization data: LCMS (>98%) *m/z* = 258.4 $[M + H]^+$ (0.766 min retention time, 254 nm). $^1$H NMR (400 MHz, $CDCl_3$): δ 6.89 (s, 2H), 6.59 (bs, 1H), 4.26 (dddd, *J* = 11.6, 10.4, 6.0, 1.6 Hz, 1H), 2.59 (d, *J* = 5.6 Hz, 1H), 2.27 (s, 3H), 2.19 (s, 3H), 2.18 (s, 3H), 1.78-1.72 (m, 2H), 1.67-1.21 (m, 8H).

**Solubility:** Solubility in PBS was determined to be 38.6 μM, which is more than 100-fold higher that the $EC_{50}$ for KCNQ2 channel activation.

**GSH Conjugates:** No glutathione conjugates detected.

**Stability:** Stability was determined for ML213 at 23°C in PBS (no antioxidants or other protectorants and DMSO concentration below 0.1%) and is shown in the table 3 and Figure 2 presented below. Due to the nature of the stability curve, it appears the instability may in fact be due to solubility issues with the compound. This can be concluded by the drop in stability between 2 hour and 24 hour and the plateau between 24 and 48 hours. However, further studies would be needed to determine this absolutely.

**Table 3.** Stability data for ML213.

| | Percent Remaining (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Compound** | **0 Min** | **15 Min** | **30 Min** | **1 Hour** | **2 Hour** | **24 Hour** | **48 Hour** |
| ML213, CID 3111211/ SID 104223736 | 100 | 89 | 98 | 89 | 83 | 47 | 43 |

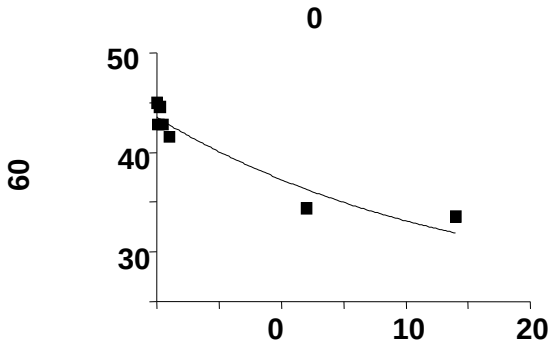



**Figure 2.** Stability data for ML213.

**Compounds added to the SMR collection (MLS#s):** 003370512 (ML213, CID 311121, 28.3 mg), 003370511 (CID 2852569, 7.3 mg), 003370510 (CID 4296446, 5.0 mg), 003370509 (CID 3843095, 8.3 mg), 003370508 (CID 4658599, 5.0 mg), 003370507 (CID 3473417, 5.2 mg).

### 2.3 Probe Preparation

***N*-mesitylbicyclo[2.2.1]heptane-2-carboxamide (ML213, CID 3111211).** To a solution of 2-*endo*-norbornanecarbonylchloride (1.0 eq, 250 mg, 1.6 mmol) in DMF (1 mL) was added 2,4,6-trimethylaniline (213 mg, 1.6 mmol) and triethylamine (440 μL, 3.2 mmol). After stirring 24 h, the reaction mixture was filtered and the supernatant purified by mass directed chromatography to provide ML213 (124.3 mg, 0.5 mmol, 30%) as a white crystalline solid. The final product was evaluated via chiral SFC (supercritical fluid chromatography) and it was shown to be a mixture of 4 diastereomers (see attached spectral analysis, Appendix 3). The *endo* starting material is the only isomer commercially available.

## 3 Results

The MLSMR library was screened for activators of KCNQ2 channels using a fluorescent assay, in which $Tl^+$ influx was measured with a fluorescent dye (FluxOR) and acted as a surrogate measure of KCNQ2 open probability. Assay data on each screening plate were normalized to generate B scores (*32*), which were used to identify 1644 hits. Confirmation experiments and counterscreening against parental cells provided a set of structural classes, which served as a basis for extensive counter-screening efforts using a combination of fluorescent and electrophysiological assays and SAR analysis of two structural classes of KCNQ2 activators. ML213 afforded potent activation of KCNQ2 channels in electrophysiological and fluorescent assays and was selective for KCNQ2 over three of the other KCNQ family members.

### 3.1 Summary of Screening Results

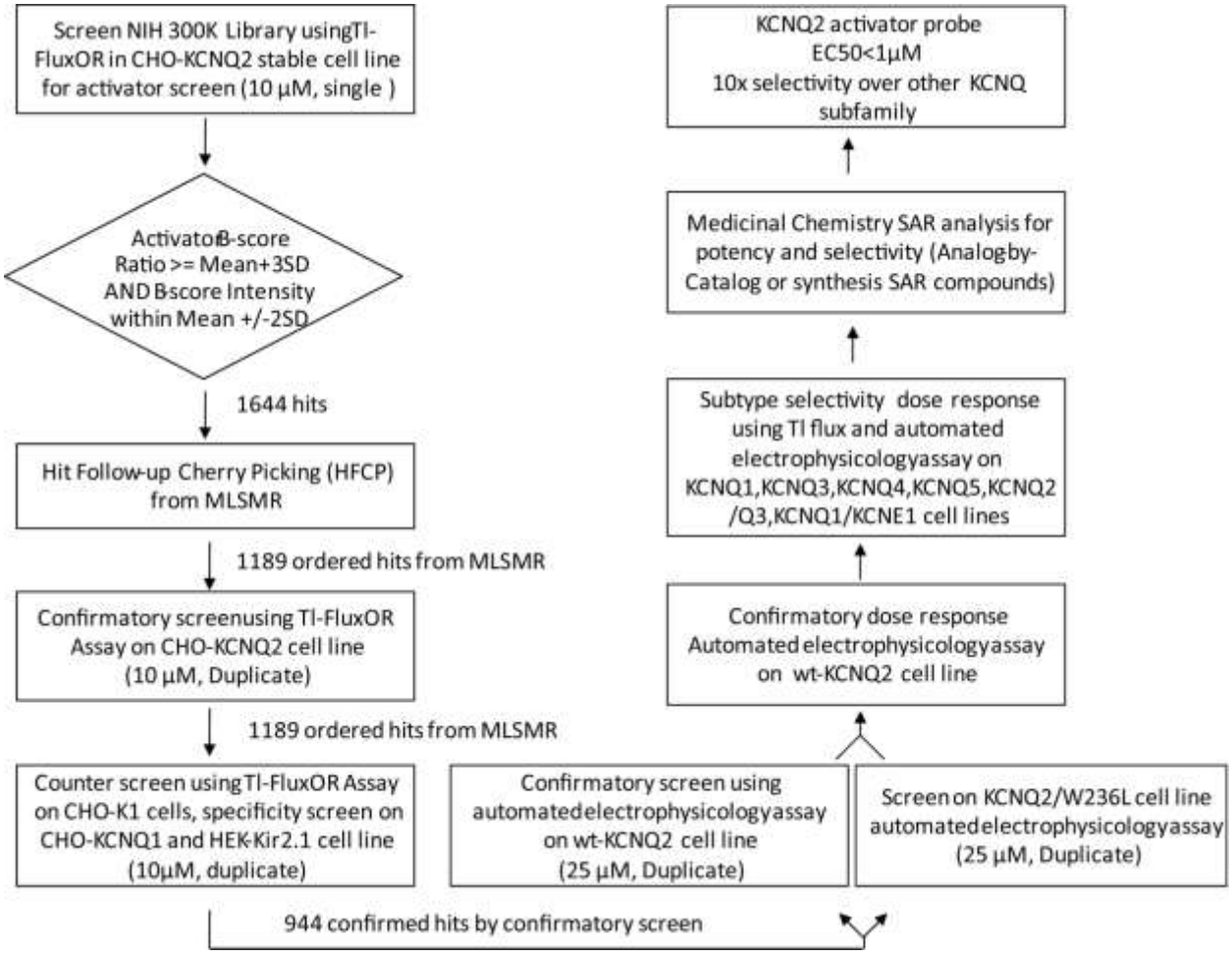



**Figure 3.** Flow chart for generation of KCNQ2 probe candidates.

Screening Summary:

1. 305k compounds initially screened in HTS assay (AID 2239) → 1644 hits
2. 1189 available compounds were retested (AID 2287) → 807 compounds validated as KCNQ2 activators using assay from primary screen
3. 1189 compounds were tested in a counterscreen against parental cells (AID 2282) → 676 compounds showed no activity in the parental cell line, yielding 468 validated KCNQ2 activator hits
4. Based on confirmatory and counter-screens, an expanded set of 944 compounds were tested on KCNQ2/W236Lchannels in an automated patch clamp assay (AID 2558) in order to possibly select for compounds with mechanisms differing from retigabine, in which 91 compounds tested as active.

5. A set of 58 compounds were selected for titration experiments in automated electrophysiology on KCNQ2 channels. 38 compounds afforded clear dose-response activation of KCNQ2 channels (AID 2603 and AID 2654).
6. From this list, Vanderbilt Specialized Chemistry Center selected two classes of compounds for SAR optimization (AID 2654).
7. CID 3111211 was identified as a probe candidate from one structural class.

### 3.2 Dose Response Curves for Probe

ML213 activation of KCNQ2 channels was evaluated in both electrophysiological and fluorescent assays. For the fluorescence assay, the activity of KCNQ2 potassium channels was monitored by the influx of a surrogate ion, $Tl^+$, for potassium. $Tl^+$ influx was detected with a fluorescent dye (FluxOR). This assay can be implemented in high-throughput formats and was used in the primary screen. As shown in **Figure 4**, ML213 produced a concentration-dependent increase in $Tl^+$ influx, with an $EC_{50}$ value of 0.36 µM and a maximal increase of 56% (**Figure 4A** and **4B**).

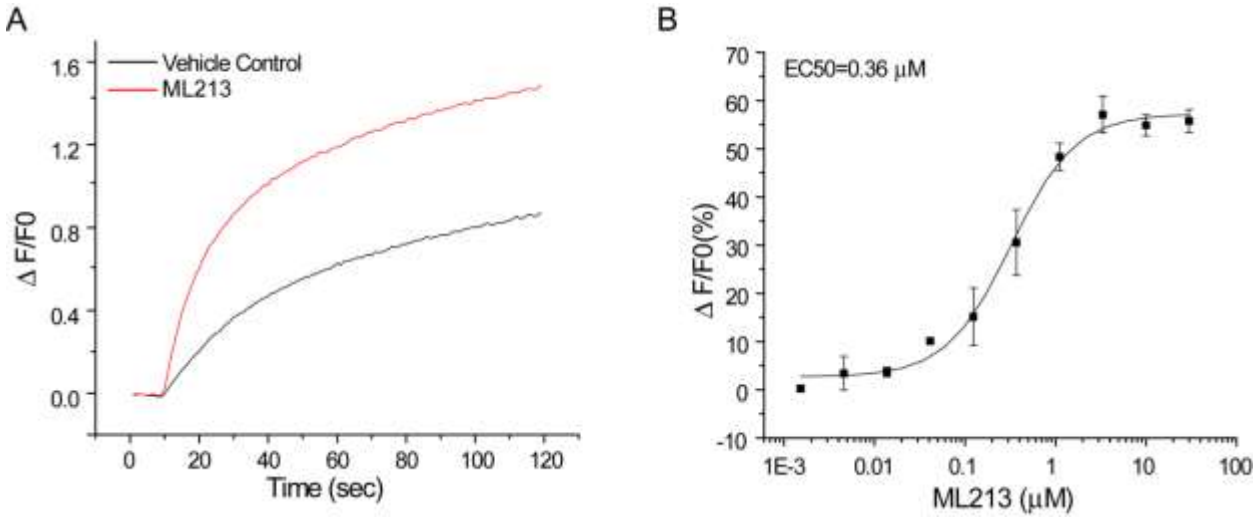



**Figure 4.** ML213 activation of KCNQ2 channels in a $Tl^+$ flux assay. (A) Typical fluorescence intensity traces in the $Tl^+$ flux assay were recorded in vehicle control (black line) and in the probe compound ML213 at 10 µM (red line). (B) Dose response curve of ML213 in the $Tl^+$ flux assay.

Currents through KCNQ2 channels were recorded using an IonWorks automated electrophysiology instrument in Population Patch Clamp mode (**Figure 5**); the results show ML213 displays a concentration dependent enhancement of KCNQ2 currents. Membrane currents provide a linear measure of channel activity and provide a direct determination of compound effects on channel function. KCNQ2 currents were activated by depolarizing voltages and after the addition of 5 µM ML213 to the KCNQ2 cells, a greater than 4-fold increase in currents was produced at -10 mV (**Figure 5A**). To evaluate $EC_{50}$ values for ML213

at different test potentials, 8-point concentration response curves for ML213 were generated at a variety of voltage steps. The $EC_{50}$ values were similar at voltages greater than -10 mV (**Figure 5B**), although the relative increases in current were decreased at higher levels of depolarization due to higher levels of channel open probability in control. At -10 mV step potential, ML213 exhibited a concentration-dependent enhancement of KCNQ2 currents with an $EC_{50}$ value of 0.23 μM and a maximal increase of 445% (**Figure 5C**). The close agreement between $EC_{50}$ values in the electrophysiological and fluorescent assays supports use of both assay methods in selectivity studies. To evaluate the mechanism for KCNQ2 potentiation by ML213 and to determine an estimate of potency that was not dependent on test potential, voltage activation curves were determined in the absence and presence of ML213. In the presence of 5 μM ML213, the $V_{1/2}$ (voltage required for half-maximal activation) of KCNQ2 was left-shifted by 37.42 ± 3.0 mV (mean±s.e.m) compared with control values in the same cells (**Figure 5D**). ML213 caused a concentration dependent shift in the $V_{1/2}$ for KCNQ2 activation with an $EC_{50}$ 0.34 ± 0.07 μM and a maximal shift of 37 mV (**Figure 5E**).

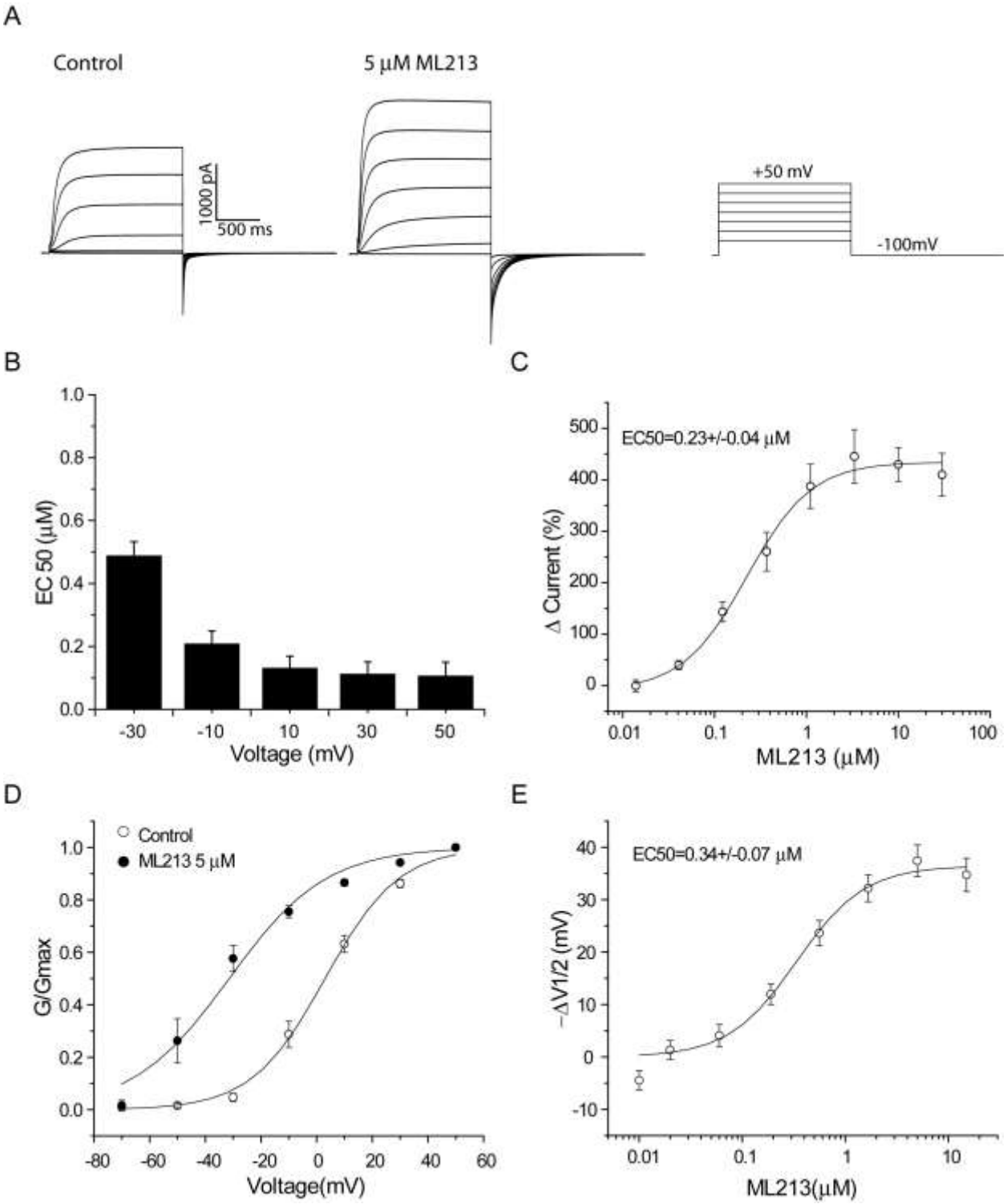



**Figure 5.** ML213 effects on voltage-dependent activation of KCNQ2 channels using automated electrophysiology. (A) KCNQ2 currents traces were recorded in the absence (left panel) and presence (middle panel) of 5 µM ML213. From a holding potential of -100mV, the membrane

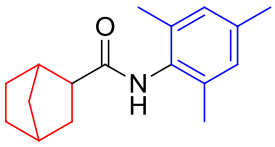
potential was changed in 20 mV incremental voltage steps from -70 mV to +50 mV (right panel). (B) $EC_{50}$ values of ML213 were determined at different voltage steps. (C) Concentration dependent activation of KCNQ2 channels measured at -10 mV. Δ Current (%) represents the percentage change in steady state current caused by ML213 compared to pre-compound measurements for each concentration. (D) KCNQ2 conductance-voltage curves were determined in the absence and presence of 5 μM ML213 and fit with Boltzmann functions to provide V1/2 (voltage of half maximal activation) values. (E) Δ V1/2 values were calculated from the differences between V1/2 in control and in ML213 in the same cells and plotted against ML213 concentration. The Δ V1/2 values were fit with a Hill equation.

### 3.3 Scaffold/Moiety Chemical Liabilities

No chemical liabilities for the probe molecule have been identified at the present time.

### 3.4 SAR Tables

Based on the distribution of KCNQ2 in the CNS, and previous work showing efficacy of KCNQ2 activators in a number of CNS related *in vivo* animal models – ML213 represents an attractive lead molecule for a CNS indication. The calculated properties of ML213 are in line with most known molecules with CNS exposure (MW < 300, cLogP = 2 – 4, tPSA <75). In addition, ML213 represents a novel chemical scaffold, as this compound does not include a heteroaryl moiety (as in ICA-27243) and displays a unique activity profile (KCNQ2 and KCNQ4 activator). Using ML213 as a starting point, the SAR evaluation was centered on the aniline portion (blue) and amide portion (red).

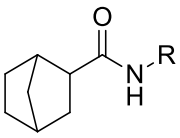


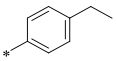
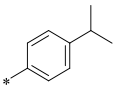
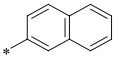

**ML 213, CID: 3111211**
MW = 257.4
cLogP = 3.66
tPSA = 29.1

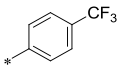
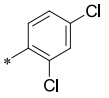
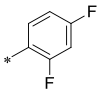
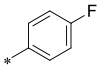
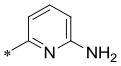
Keeping the bicyclo[2.2.1]heptylamide constant, a variety of anilines were investigated. The 2,4-dimethyl derivative (SID 103911606) was equipotent with ML213 (220 nM; 194% maximal change). Moving the methyl groups around the ring revealed interesting SAR, the 2,5-dimethyl compound (SID 103073347) was also equipotent (400 nM; 251% maximal change), but the 2,6-dimethyl and 2,3-dimethyl derivatives were at least 3 to 10-fold less potent (3,240 nM; 198% maximal change and 1,000 nM; 426% maximal change, respectively). Moving the dimethyl groups to the meta positions (3,5-dimethyl) as in SID 103911620 led to a much less active compound (47.4% maximal change at 30 μM). Removal of two of the methyl groups, leaving the 4-substituted compound retained activity (SID 103911615, 750 nM; 167% maximal change).

The simple phenyl derivative also showed a potentiation of the maximal change (136% at 30 μM; $EC_{50}$ not determined). Extending the substitution in the 4-position to either an ethyl group (SID 103911605, 920 nM; 305% maximal change), an isopropyl group (SID: 103911608, 720 nM; 191% maximal change) or a 2-naphthyl group (SID 103911607; 600 nM; 143% maximal change) were all tolerated. Interestingly, replacing the 4-methyl with a 4-trifluoromethyl group led to an inactive compound (SID 103911618). Finally, addition of halogens (2,4-dichloro, SID 103911621; 1,520 nM, 171% maximal change; 2,4-difluoro, SID 103911617, 10,050 nM, 147% maximal change; 4-fluoro, SID 103911616, 61% maximal change, $EC_{50}$ not determined) was not tolerated. Replacement of the phenyl with a pyridyl was also not tolerated (SID 103911604, inactive), although a substituted pyridyl was active (SID 103911624, 1,810 nM, 500% maximal change).

**Table 4.** SAR Evaluation of the right-hand portion of ML213.



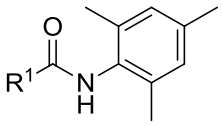

| Entry | SID | Synthesis/ Catalog | R | KCNQ2 $EC_{50}$ (μM)[a] (% at 30μM)[b] | Maximal Change (%) |
|---|---|---|---|---|---|
| 1 | SID 104223736 | S |  | 0.33 ± 0.11[c] | 421.4 ± 148.2 |
| 2 | SID 103911606 | S |  | 0.22 ± 0.07 | 193.8 ± 8.7 |
| 3 | SID 103073347 | C |  | 0.40 ± 0.13 | 251.2 ± 17.4 |
| 4 | SID 103073348 | C |  | 3.24 ± 1.0 | 197.7 ± 22.8 |
| 5 | SID 103911605 | S |  | 0.92 ± 0.25 | 305.0 ± 43.1 |
| 6 | SID 103911608 | S |  | 0.72 ± 0.19 | 190.8 ± 16.7 |
| 7 | SID 103911615 | S |  | 0.75 ± 0.29 | 167.3 ± 11.5 |
| 8 | SID 103911623 | S |  | 1.00 ± 0.47 | 426.3 ± 64.9 |
| 9 | SID 103911607 | S |  | 0.60 ± 0.19 | 142.7 ± 19.7 |
| 10 | SID 103911619 | S |  | (135.9) | 135.9 ± 27.8 |
| 11 | SID 103911620 | S |  | (47.4) | 47.4 ± 8.0 |

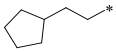
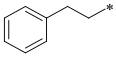
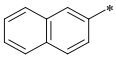
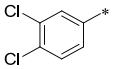
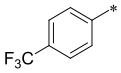
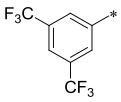
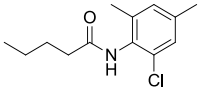
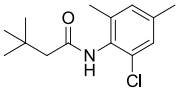
| | | | | | |
|---|---|---|---|---|---|
| 12 | SID 103911618 | S |  | Inactive | |
| 13 | SID 103911621 | S |  | 1.52 ± 0.20 | 170.7 ± 22.9 |
| 14 | SID 103911617 | S |  | 10.05 ± 2.49 | 147.1 ± 16.9 |
| 15 | SID 103911616 | S |  | (61.0) | 61.0 ± 7.0 |
| 16 | SID 103911624 | S |  | 1.81 ± 0.62 | 499.9 ± 84.95 |
| 17 | SID 103911604 | S |  | Inactive | |

[a] $EC_{50}$'s were generated from 8-point of concentration response curves with 3-fold dilutions starting from the maximal concentration (30μM) with quadruplicates. Activity definition: The compound will be defined as inactive if the compound exhibits less than 30% activation at 30μM. Otherwise, the compound will be defined as an activator with the calculated $EC_{50}$ value. [b] In cases in which a saturating response was not obtained at the highest tested concentration, (% at 30μM) is listed [c] $EC_{50}$ values were obtained from five sets of experiments with quadruplicate measurements for each experiment. $EC_{50}$ values are expressed as $EC_{50} \pm$ SD, using estimated standard deviations provided by the fitting software (Origin 6.0).

The next round of SAR kept the mesityl (2,4,6-trimethyl) aniline constant and a variety of alky, cycloalkyl and aryl amides were evaluated. Groups replacing the bicyclo[2.2.1]heptane were not well tolerated. Replacement of the bicyclo[2.2.1]heptane group with a simple cyclohexyl group (i.e., removal of the bridgehead group) led to a 5-fold loss in activity (1,570 nM; 230% maximal change). Simple alkyl or branched alkyl groups were not tolerated (SID: 103911622, 103% maximal change, $EC_{50}$ not determined; SID 103911597, 79% maximal change, $EC_{50}$ not determined; SID 103911596, 38% maximal change, $EC_{50}$ not determined), nor was the bulkier adamantyl group (SID 103911602, inactive). The only group that gave comparable activity to ML213 was the cyclopentylethyl group (SID 103911613, 490 nM, 310% maximal change). Replacement with aryl groups or substituted aryl groups led to inactive compounds. It is recognized that ML213 is a mixture of diastereomers, although this probe should still be a valuable tool for in vitro experiments.

**Table 5.** SAR Evaluation of the left-hand amide portion of ML213.



| Entry | SID | Synthesis/ Catalog | $R^1$ | KCNQ2 $EC_{50}$ (μM)[a] (% at 30μM)[b] | Maximal Change (%) |
|---|---|---|---|---|---|
| 18 | SID 103911599 | S |  | 1.57 ± 0.30[c] | 230.0 ± 24.1 |
| 19 | SID 103911622 | S |  | (103.1) | 103.1 ± 15.3 |
| 20 | SID 103911597 | S |  | (79.0) | 79.0 ± 4.4 |
| 21 | SID 103911596 | S |  | (38.0) | 38.0 ± 4.1 |
| 22 | SID 103911602 | S |  | Inactive | |
| 23 | SID 103911611 | S |  | Inactive | |
| 24 | SID 103911613 | S |  | 0.49 ± 0.23 | 310.3 ± 37.3 |
| 25 | SID 103911614 | S |  | 1.60 ± 0.72 | 236.7 ± 48.9 |
| 26 | SID 103911598 | S |  | Inactive | |
| 27 | SID 103911601 | S |  | Inactive | |
| 28 | SID 103911609 | S |  | (31.8) | 31.8 ± 6.8 |
| 29 | SID 103911603 | S |  | (57.3) | 57.3 ± 23.8 |
| 30 | SID 103911610 | S |  | (32.3) | 32.3 ± 6.4 |
| 31 | SID 103911612 | S |  | Inactive | |
| 32 | SID 103911600 | S |  | 0.60 ± 0.12 (Inhibitor) | –47.8 ± 1.6 |
| 33 | SID 103073349 | C |  | 2.1 ± 0.6 | 314.3 ± 52.5 |
| 34 | SID 103073350 | C |  | 0.87 ± 0.2 | 318.1 ± 19.9 |

[a] $EC_{50}$'s were generated from 8-point of concentration response curves with 3-fold dilutions starting from the maximal concentration (30μM) with quadruplicates. Activity definition: The compound will be

defined as inactive if the compound exhibits less than 30% activation at 30μM. Otherwise, the compound will be defined as an activator with the calculated $EC_{50}$ value. [b] In cases in which a saturating response was not obtained at the highest tested concentration, (% at 30μM) is listed [c] $EC_{50}$ values were obtained from five sets of experiments with quadruplicate measurements for each experiment. $EC_{50}$ values are expressed as $EC_{50} \pm$ SD, using estimated standard deviations provided by the fitting software (Origin 6.0).

### 3.5 Cellular Activity

All assays for KCNQ2 and other ion channels described in this report are cell-based assays. The activity of ML213 in these assays indicates that the probe compound exhibits adequate cell permeability to support further studies with native cells or tissues. No acute toxicity was observed in the cell-based assays.

### 3.6 Profiling Assays

ML213 (CID 3111211) has been tested in 247 assays performed within the MLPCN network and was active in only one assay (Cycloheximide Counter-screen for Small Molecule Inhibitors of Shiga Toxin [Primary Screening: AID 2314]) that was not dependent on KCNQ2.

ML213 was evaluated in fluorescent and electrophysiological assays against six related channels from the KCNQ family and against two distantly related potassium channels, hERG and Kir2.1. The results are listed in **Table 6**. For comparison, CID 3111211 displayed $EC_{50}$ values for KCNQ2 activation of 0.36 μM in a fluorescent $Tl^+$ influx assay and 0.23 μM in an IonWorks electrophysiological assay.

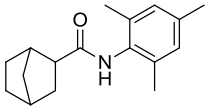
**Table 6.** Selectivity of ML213 for activation of KCNQ2 channels compared with activation or block of other potassium channels. $EC_{50}$ values are calculated from dose-dependent activation curves for KCNQ channels and for inhibition of Kir2.1 and hERG channels.

| Channel | AID ($Tl^+$ $EC_{50}$, μM) | Fold selectivity | AID (IW $EC_{50}$, μM) | Fold selectivity |
|---|---|---|---|---|
| KCNQ1 | AID 504417 (>30) | >80 | AID 493044 (>30) | >80 |
| KCNQ1/E1 | AID 504418 (>30) | >80 | AID 493042 (>30) | >80 |
| KCNQ2/3 | | | AID 493039 (0.37) | 1 |
| KCNQ3 | AID 493047 (>30) | >80 | | |
| KCNQ4 | AID 504416 (2.4) | 6.7 | AID 493043 (0.51) | 2.2 |
| KCNQ5 | AID 493046 (>30) | >80 | | |
| Kir2.1 | AID 2345 (>10) | >27 | | |
| hERG | | | (>10) | >28 |

To more fully characterize this novel KCNQ2 and KCNQ4 probe molecule, ML213 was tested on Ricerca's (formerly MDS Pharma's) Lead Profiling Screen (binding assay panel of 68 GPCRs, ion channels and transporters screened at 10 μM), and was found to not significantly bind with any of the 68 assays conducted (no inhibition of radio ligand binding > 50% at 10 μM). Thus, from the Ricerca selectivity profile, inactivity in the PubChem assays and the selectivity versus closely related ion channels in which ML213 affords greater than 25-fold selectivity for

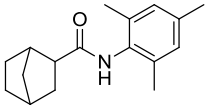
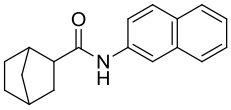
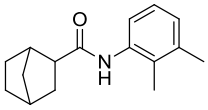
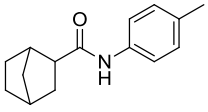
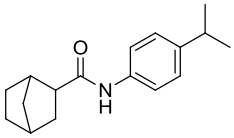
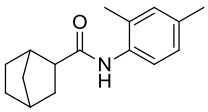
activation or block of related KCNQ channels and other distantly related potassium channel except for KCNQ4 channels, ML213 (CID 3111211) is highly selective and can be used to dissect the role of KCNQ2 and KCNQ4 activators *in vitro* and, potentially, *in vivo*.

**Table 7.** Ricerca Profiling of SID 104223736/VU0448090-1.

| SID/VU# | Structure | Primary Biochemical Assay | Species | % inhibition @ 10 μM |
|---|---|---|---|---|
| SID 104223736/ VU0448090-1 |  | *No Significant Activity Found* | | |

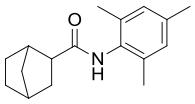
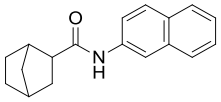
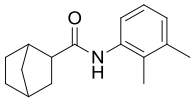
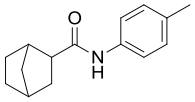
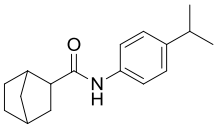
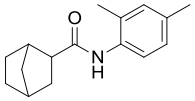
ML213 and other analogs were also evaluated in our tier 1 *in vitro* DMPK battery to further establish its utility as a small molecule probe. In $CYP_{450}$ assays, ML213 was clean against all of the CYP enzymes evaluated (>30 μM). Other analogs of ML213 also displayed a favorable profile against CYP inhibition with the exception of SID 103911607, which showed inhibition of CYP1A2 (830 nM) and CYP2C9 (7,680 nM) and SID 103911623, which inhibited CYP1A2 (7,750 nM). All other compounds tested were >10 μM against all CYPs.

**Table 8.** $CYP_{450}$ Inhibition for ML213 and analogs

| SID/VU# | Structure | CYP450 (μM) 1A2 | 2C9 | 2D6 | 3A4 |
|---|---|---|---|---|---|
| SID 104223736/ VU0448090-1 |  | >30 | >30 | >30 | >30 |
| SID 103911607/ VU0447127-1 |  | 0.83 | 7.68 | >30 | >30 |
| SID 103911623/ VU0447138-1 |  | 7.75 | >30 | >30 | 22.22 |
| SID 103911615/ VU0447134-1 |  | 10.37 | >30 | >30 | >30 |
| SID 103911608/ VU0000760-1 |  | 12.23 | 27.04 | >25 | >30 |
| SID 103911606/ VU0447126-1 |  | 10.51 | >30 | >30 | >30 |

The metabolic stability of ML213 and analogs were evaluated in assays which can predict rat and human hepatic clearance from *in vitro* microsomal clearance values (Table 9). This allows for a rank ordering of compounds that would be predicted to have poor stability in *in vivo* testing protocols (after oral dosing). Unfortunately, all of the compounds evaluated showed high (near hepatic blood flow) clearance in human and rat liver microsomes. This result is not surprising as all the compounds tested contain an unsubstituted benzylmethyl or naphthyl group which is known to undergo oxidation by liver microsomes. However, ML213 did display an acceptable free fraction profile (6.5% and 1.2% free unbound for human and rat, respectively). Other analogs also showed free fraction profile >1-3% as well.

**Table 9.** Intrinsic clearance and protein binding data of ML213 and analogs.

| Compound SID/VU# | Structure | Intrinsic Clearance Human $CL_{INT}$ | Intrinsic Clearance Human $CL_{HEP}$ | Intrinsic Clearance Rat $CL_{INT}$ | Intrinsic Clearance Rat $CL_{HEP}$ | Protein Binding (% fu)* Human | Protein Binding (% fu)* Rat |
|---|---|---|---|---|---|---|---|
| ML213/ SID 104223736/ VU0448090-1 |  | 64.8 | 15.9 | 645.1 | 63.1 | 6.5 | 1.2 |
| SID 103911607/ VU0447127-1 |  | 68.4 | 16.1 | 406.2 | 59.7 | 0.5 | 0.2 |
| SID 103911623/ VU0447138-1 |  | 55.9 | 15.3 | 691.9 | 63.6 | 6.2 | 4.4 |
| SID 103911615/ VU0447134-1 |  | 41.9 | 14.0 | 277.6 | 55.9 | 4.7 | 3.9 |
| SID 103911608/ VU0000760-1 |  | 42.4 | 14.0 | 1659.0 | 67.2 | 0.6 | 0.7 |
| SID 103911606/ VU0447126-1 |  | 36.5 | 13.3 | 496.9 | 61.4 | 6.4 | 2.0 |

This data suggests that ML213 would likely not be appropriate for oral dosing; however, several other dosing options are available for compounds that undergo significant first-pass metabolism (e.g., subcutaneous dosing, intravenous dosing). Thus, due to the therapeutic importance and distribution of KCNQ2 and KCNQ4 in the CNS, we further profiled ML213 in a snapshot *in vivo* PK study to assess the plasma and brain levels after a single time point (1 hr, 10 mg/kg) following subcutaneous administration (**Table 10**). ML213 displayed plasma levels after 1 hour time point greater than 1 μM (1.03 μM); however, ML213 displayed poor brain levels at the

same time point (0.095 μM); resulting in a very low B/P (0.09). ML213 does exhibit lower solubility than desired (~40 μM) and this could be contributing to the low brain levels. Further optimization of ML213 is planned in order to address the issues of metabolic stability and solubility in order to refine the *in vivo* PK properties.

**Table 10**. Brain:plasma ratio after subcutaneous administration in rat.

| SC – ML213 (1 hr) | | | | | |
|---|---|---|---|---|---|
| Dose (mg/kg | Plasma Systemic (ng/mL) | Plasma Systemic (μM) | Brain (ng/g) | Brain (μM) | Brain:Plasma |
| 10 | 265 | 1.03 | 24.4 | 0.095 | 0.09 |

**Table 11:** Calculated Property Comparison of ML213 with MDDR Compounds

| Property | ML213, CID3111211 | MDDR Phase I | MDDR Launched |
|---|---|---|---|
| MW | 257.4 | 438.98 | 415.20 |
| cLogP | 4.11 | 3.21 | 2.21 |
| TPSA | 29.1 | 97.06 | 91.78 |
| Hdon | 1 | 2.12 | 2.13 |
| Hacc | 1 | 7.06 | 6.47 |
| LogS | -4.23 | -4.96 | -3.73 |
| NrotB | 2 | 7.08 | 5.71 |

# 4 Discussion

## 4.1 Comparison to existing art and how the new probe is an improvement

A number of structural classes of compounds have been described in the scientific and patent literature that act as KCNQ channel activators (*vide supra*) (*2, 11, 31*). Broad selectivity analysis has not been presented for many described KCNQ-activating compounds. BMS-204352 and retigabine have been evaluated for activation of KCNQ2, KCNQ2/3, KCNQ3/4 and KCNQ4 channels and both compounds exhibit similar levels of activation for all channel subtypes (*10, 33-35*). More recent examples from Bristol-Myers Squibb also have been reported as pan-KCNQ activators; although the potency against the other members of the family has not been reported. Icagen's most recent compound (ICA-27243) shows selectivity for KCNQ2/Q3 versus KCNQ4 (25-fold) and KCNQ3/Q5 (>75-fold), although data against KCNQ1 was not reported. Compound **6** is selective for KCNQ2/Q3 versus KCNQ1 + KCNE1; however, no other data have been reported. Selectivity data is lacking for other compounds disclosed in the patent literature.

In contrast to other reported activators, ML213 exhibits nanomolar activation of KCNQ2 and KCNQ4 channels, and exhibits selectivity against other subtypes (>80 fold selective versus KCNQ1, KCNQ3, KCNQ5, KCNQ1 + KCNE1). In addition, ML213 was inactive against a profiling assay of 68 GPCRs, transporters and ion channels (Ricerca Lead Profiling), and was 27-fold selective against another potassium channel (Kir2.1). ML213 also shows an excellent profile against the major CYP enzymes (>80-fold selectivity). Unfortunately, ML213 shows poor stability in liver microsomes and suboptimal brain levels after subcutaneous dosing. However, ML213 represents the most potent and selective KCNQ2/Q4 activator to date.

### 4.2 Mechanism of Action Studies

The anticonvulsant, retigabine, activates KCNQ2 and KCNQ2/3 heteromultimeric channels. A single amino acid substitution, W236L, removes sensitivity of KCNQ2 channels to retigabine activation (*34, 35*). ML213 was evaluated for effects on KCNQ2/W236L channels to evaluate the possibility of a shared binding site or mechanism with retigabine. ML213 was inactive on KCNQ2/W236L channels, suggesting possible similar interaction sites with retigabine. ML213 largely shifts the conductance-voltage curve to hyperpolarizing potentials. The combination of increased conductance and hyperpolarizing shift of half maximal activation contributes to the overall augmentation of current amplitude.

### 4.3 Planned Future Studies

ML213 is suitable for *in vitro* studies of the functional properties and roles of KCNQ2 and KCNQ4 channels. These studies could include, but are not limited to, evaluation of the roles of these channels in controlling excitability in isolated neurons and in tissue slices. Since mutations in KCNQ2 channels are involved in some forms of human epilepsy, ML213 would be useful for examining mechanisms underlying epileptogenesis in tissue slices. ML213 is unique in displaying selectivity for activation of KCNQ4 over KCNQ3 and KCNQ5 channels. KCNQ4 channels are expressed in neurons in the auditory system and ML213 may provide a tool to investigate the role of these channels in auditory processing. ML213 may be a useful probe for mapping channel domains involved in gating by constructing chimeric channels composed of domains from related KCNQ channels with differing sensitivities to activation by ML213. With respect to improving the liver metabolic profile, more expanded SAR may be applied. However, our data have clearly shown that benzylmethyl or naphthyl group cannot be replaced.

Improvements in the metabolic properties of ML213, while retaining or improving potency and selectivity, would provide a useful probe for in vivo analysis of the roles of KCNQ2 and KCNQ4 channels in CNS function. In addition, the diastereomeric ratio will be further evaluated in the extended probe phase of the project. Chemistry will be developed to synthesize and characterize each of the isomers; or, the [2.2.1]-bicyclo will be eliminated altogether in order to improve on the calculated properties and minimize the lipophilicity of the molecule.

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# 6 Appendix

## Appendix 1. Cell-based FDSS $Tl^+$ flux assay

1) Cell culture: Cells are routinely cultured in DMEM/F12 medium, supplemented with 10% Fetal Bovine Serum (FBS), 50 IU/ml penicillin, 50 µg/ml streptomycin, and 500 µg/ml G418.
2) Cell plating: Add 50 µl/well of 120,000 cells/ml re-suspended in DMEM/F12 medium with 10% FBS.
3) Incubate overnight at 37$^o$C and 5% $CO_2$.
4) Remove medium and add 25 µl/well of 1x FluxOR solution to cells.
5) Incubate 90 minutes at room temperature (RT) in darkness.
6) Prepare 7.5x compound plates and control plates on the Cybi-Well system: test compounds are prepared using assay buffer; controls are assay buffer (IC0), ICmax of ZTZ240(*36*) (all with DMSO concentrations matched to that of test compounds).
7) Remove FluxOR dye solution and add 20 µl/well of assay buffer to cells.
8) Add 4 µl of 7.5x compound stock into the cell plates via Cybi-Well system.
9) Incubate all cell plates for 20 minutes at RT in darkness.
10) Prepare 5x stimulus buffer containing 12.5 mM $K_2SO_4$ and 12.5 mM $Tl_2SO_4$.

11) Load cell plates to Hamamatsu FDSS 6000 kinetic imaging plate reader.
12) Measure fluorescence for 10 seconds at 1Hz to establish baseline.
13) Depolarize cells with 6 µl/well of stimulus buffer and continue measuring fluorescence for 110 seconds.
14) Calculate ratio readout as F(max-min)/F0.
15) Calculate the average and standard deviation for negative and positive controls in each plate, as well as Z and Z' factors.
16) Calculate B scores for test compounds using ratios calculated in Step 14.
17) Outcome assignment: If the B score of the test compound is more than 3 times the standard deviation (SD) of the B scores of ratios of the library compounds (>=3*SD), AND the B score of initial fluorescence intensity is within 3 times the standard deviation of the B scores of the library compounds, the compound is designated in the Outcome as an active (Value=2) potentiator of the KCNQ2 channels. Otherwise, it is designated as inactive (value=1).
18) Score assignment: An active test compound is assigned a score between 0 and 100 by calculation of INT(100*LOG(B Score Potentiator Ratio), they are normalized to the smallest and largest LOG(B Score Potentiator Ratio), B Score Potentiator Ratio.

**Appendix 2. <u>KCNQ potassium channel IonWorks electrophysiology assay</u>**

KCNQ2 activity was examined in an electrophysiological assay using the population patch clamp mode on the Ionworks Quattro (MDC, Sunnyvale, CA), an automated patch clamp instrument. The CHO cells stably expressing KCNQ2 channels were freshly dislodged from flasks and dispensed into a 384-well population patch clamp (PPC) plate. The cell plating density was 7,000 cells/well suspended in the extracellular solution, composed of (in mM): 137 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4 adjusted with NaOH.

After dispensing, seal resistance of cells was measured for each well and cells were perforated by incubation with 50µg/ml amphotericin B (Sigma, St. Louis, MO), which was dissolved in the internal solution composed of (in mM): 40 KCl, 100 K-Gluconate, 1 $MgCl_2$, 5 HEPES, 2 $CaCl_2$, pH 7.2 adjusted with KOH. Activity of KCNQ2 was then measured with the recording protocol as followings. Leak currents were linear subtracted extrapolating the current elicited by a 100-ms step to -100 mV from a holding potential of -90 mV. During the voltage pulse protocol, cells were held at -90 mV, followed by by 2,000 ms depolarizing step from -90 mV to -10 mV, and then back to -90mV for 2000 ms. The currents were measured at the end of the depolarization pulse before and after the application of compounds for 3 min. Only cells with a current amplitude more than 100 pA at -10 mV and a seal resistance >30 MΩ were included in the data analysis.

Compound effects were assessed by the percentage changes in the KCNQ2 steady state currents, which were calculated by dividing the difference between pre- and post-compound KCNQ2 currents by the respective pre-compound currents in the same well.

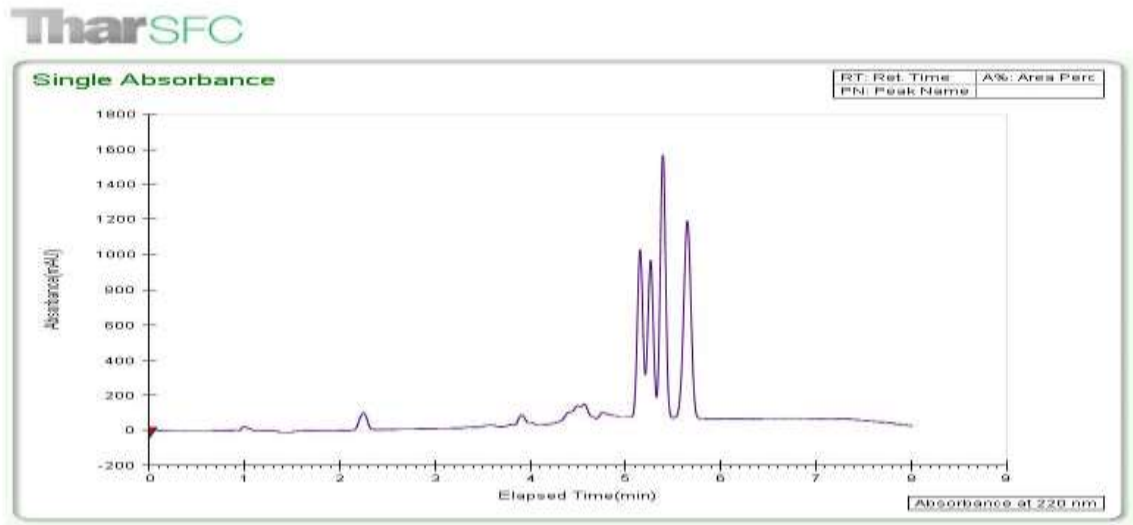
When constructing conductance-voltage curves, conductance values were calculated by dividing the steady state outward currents measured during the voltage steps by the driving force (step voltage minus the calculated potassium reversal potential).

The KCNQ2 protocol was also used for KCNQ2/KCNQ3 recording. But for KCNQ1 and KCNQ4, the cells were depolarized to +40mV from the holding potential -70 mV. Currents were measured at the step current at +40 mV. And for KCNQ1/KCNE1, cells were stimulated by 3,000 ms depolarizing step from -70 mV to +40 mV, followed by hyperpolarization to -20 mV for 500 ms. Currents were measured at the steady state of +40mV voltage step.

No corrections for liquid junction potentials (estimated as -20 mV by comparing the KCNQ2 reversal potential with the calculated Nernst potential for potassium) were applied. The current signal was sampled at 0.625 kHz.

**Appendix 3.**

ML213 was in parallel resynthesized using two commercially available starting materials, an “endo-prefering” carboxylic was used to make SDT-I-35 and an “endo” carboxylic acid was used to make SDT-I-117. The products were analyzed by chiral supercritical fluid chromatography (SFC) analysis (see below). Both products exhibited multiple peaks indicating that both products were composed of similar mixtures of diasteromers.




| General Info | | | |
|---|---|---|---|
| Log Author | | Report Date | 6/22/2011 |
| Log Date | 6/22/2011 4:06:56 PM | Method Name | 5_3D_gradient.met |
| Report By | current_User | Notes | |

| Injection Info | | | |
|---|---|---|---|
| Inj Vol | 10 | Temp | 40 |
| Solvent | EtOH 0.1% DEA | Flow | 3.5 |
| Column | Lux Cellulose-4 | % Modifier | 5 |
| Sample | SDT-I-117 | Pressure | 100 |
| Well location | P1: 1C | | |

TharSFC
C:\SFC\Data\General\SDT-I-117_6-22-2011_4_06_56 PM.tta

**Figure A1.** SFC analysis of STD-I-117

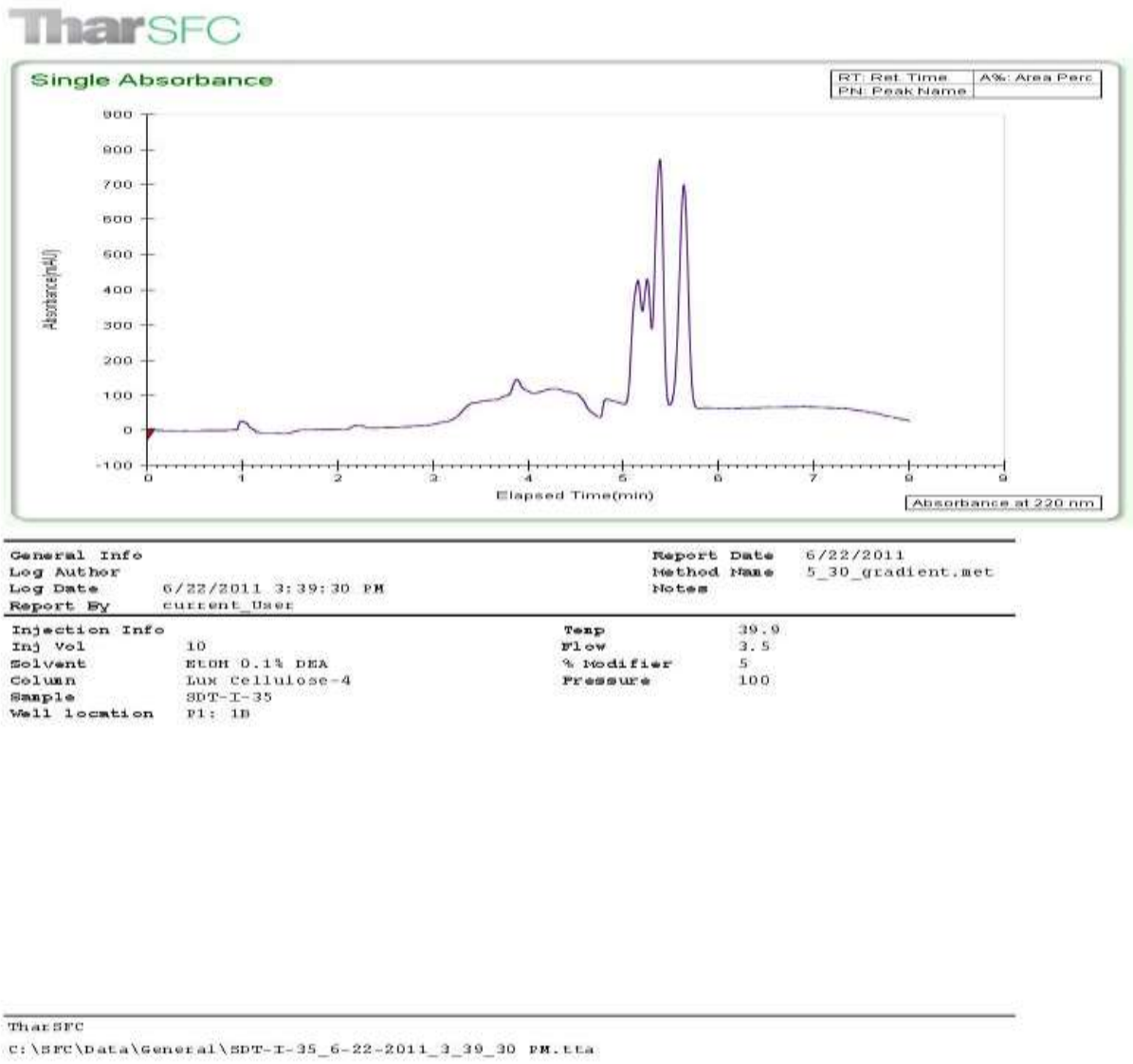



**Figure A2.** SFC analysis of SDT-I-35